The Effect of Donor Sex on Influenza A Infection in Primary Human Nasal Epithelial Cell Cultures

by

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ABSTRACT

Influenza viruses cause disease in millions of individuals worldwide per year through seasonal epidemics and, less frequently, through avian influenza outbreaks and pandemics. Generally, women of reproductive ages experience greater morbidity and mortality when compared to their male counterparts during these outbreaks. Although sex and gender have been considered in epidemiological, clinical, and animal studies of infection and vaccination, no studies have considered whether the donor sex of cell cultures affects the response to infection with influenza A virus. To better understand and characterize the sex differences in response to influenza A virus infection, we infected primary, differentiated human nasal epithelial cell (hNEC) cultures from male and female donors with recombinant influenza A/Udorn/307/72 H3N2 virus. We tested the hypothesis that hNECs from male and female donors would replicate virus to the same titer as well as have a different innate immune response to infection. Following infection, viral infection kinetics and titers were similar in hNEC cultures from male and female donors. hNEC cultures from female donors produced more IFN-λ than their male counterparts while hNEC cultures from male donors produced higher concentration of several chemokines. Cultures derived from males also displayed greater antiviral transcriptional activity. In summary, these data suggest that hNECs from male donors have a greater innate immune response to infection than females, but this does not impact the ability to control infection. These data provide novel insights into how the sex of a donor affects cellular responses to infection.

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TABLE OF CONTENTS

LIST OF TABLES

Tables

LIST OF FIGURES

Results

INTRODUCTION

Influenza Virus Biology

Influenza is caused by members of the *Orthomyxoviridae* family of viruses: influenza A, B, and C. Influenza A viruses (IAV) are the most common cause of influenza in humans and induce the most severe symptoms (WHO, 2015). The IAV genome is comprised of eight, negative-sense, RNA segments that encode for 11 proteins, including matrix proteins (M) 1 and 2, nonstructural protein (NS) 1 and polymerase basic (PB) 1, which are important for structure, function, and dissemination of the virus. The surface proteins hemagglutinin (HA) and neuraminidase (NA) are the immunodominate antigens; the combination of HA and NA determines the strain of influenza (Lagace-Wiens et al., 2010; Gorman et al., 1991). Selective pressure from the host immune system drive the evolution of the surface glycoproteins of the virus enabling the virus to infect individuals with new strains despite previous influenza infections (Gorman et al., 1991). Single point mutations in the RNA amino acid sequence of the virus result in antigenic drift (Jagger et al., 2012) while coinfection of a single host with two different viruses can result in genetic reassortment of the virus genome, termed antigenic shift (Gorbach et al., 2014). Both antigenic shift and drift are responsible for the eighteen HA and eleven NA glycoproteins that have been identified of which 3 HA (H1, H2, H3) and two NA (N1 and N2) are responsible for human pandemics (Gorbach et al., 2014). These genetic reassortments of the virus have the ability to produce novel influenza viruses capable of causing the next epidemic or pandemic in a naïve population.

The hemagglutinin of IAVs is a homotrimer that forms spikes on the surface of the virion, allowing it to bind to the sialic acid receptors on the surface of the host cell membrane (Nayak et al., 2009). These sialic acid receptors, α(2,3) and α(2,6), confer species specificity with human IAVs generally recognizing the α(2,6) formation while avian IAVs preferentially utilize α(2,3). Swine IAVs can form linkages with both α(2,3) and α(2,6) receptors making it an ideal vessel for viral recombination (Samji, 2009). During viral release from an infected cell, NA is responsible for cleaving the IAV bond with the sialic acid receptor, aiding in the release of viral progeny from the cell (Nayak et al., 2009). The inherent virulence of IAV is a major determinant of the severity of illness associated with infection (WHO, 2015).

Influenza A viruses undergo several steps after binding to its preferred sialic acid receptor in order to transfer its genetic material and ultimately replicate. After the virus enters the cell via endocytosis, the vesicle it is contained within fuses with a host endosome. Once the viral and endosome membranes fuse, the drop in pH allows for conformation changes of the HA protein; this then disrupts interact between the viral ribonucleoproteins (vRNPs) and the M1 and M2 proteins which allows the vRNPs to enter the cytoplasm (Yoshimura et al., 1982). The vRNPs then travel to the nucleus where they are taken up and the transcriptase contained within the vRNPs transcribes the viral RNA into mRNA, which can then be used to synthesize new viral proteins. The new virions are packaged and then bud from the cell surface from which they initially attached. Viral NA cleaves the sialic acid receptor to release the new virion back into the respiratory tract (Rapid Reference, 2006).

Infection of Epithelial Cells by Influenza Viruses

In humans, seasonal strains of IAV infect ciliated epithelial cells in the nasal cavity, trachea, and bronchi,--components of the upper and lower respiratory tracts (Eccles, 2005). Avian strains of IAV, such as H5N1 and H7N2, typically infect only the lower respiratory tract, such as the alveolar type 2 pneumocytes and club cells of the bronchi (Short et al., 2014). These differences in infection sites impact the severity of disease. Uncomplicated cases of seasonal influenza are generally associated with infection limited to the upper respiratory tract, with nonspecific symptoms, including cough, sore throat, fever, and myalgia, arising from inflammation of the respiratory system. In the lower respiratory tract, the virus can damage these very sensitive tissues that are necessary for gas exchange, including type 1 and 2 pneumocytes, leading to respiratory difficulties and more severe disease (Short et al., 2014). Complicated cases of seasonal influenza most often occur in individuals with compromised immune systems or those suffering from other co-morbidities, such as chronic obstructive pulmonary disease (COPD) (WHO, 2015). However, healthy individuals can present with similar complications from IAV infections, especially when infected with pandemic or epidemic strains of the virus (Short et al., 2014).

Data on the interaction of IAVs with mouse and human epithelial cells demonstrate that the presence of sialic acid receptors on epithelial cells determines susceptibility to IAV infection. A study of cell tropism and cell susceptibility to IAV

infection demonstrated differences in virus-specific receptor location in humans as compared with mice. Ciliated mouse tracheal epithelial cells (mTECs) and human tracheal epithelial cells (hTECs) were found to both express α2,3-sialic acid receptors making them both susceptible to infection with mouse adapted recombinant influenza A/WSN/33 H1N1 (rWSN) (Yan et al., 2016). In contrast, infection with A/California/7/2009 H1N1 (2009 H1N1) productively infected both ciliated and nonciliated hTECs expressing the α2,6-sialic acid receptor. Importantly, mTECS lack the α2,6-sialic acid receptor and, therefore, 2009 H1N1 could not infect mTECs (Yan et al., 2016).

To further examine the ability of human strains to infect cells from mice and humans, the human seasonal lab-adapted IAV strain, A/Udorn/72 H3N2 rUdorn H3N2 was introduced into mTEC and hTEC culture. The H3N2 rUdorn virus was able to infect both ciliated and nonciliated cells in the hTECs using both α2,6 and α2,3 receptors, whereas rUdorn could only infected ciliated cells expressing the α2,3 receptor in the mTECs (Yan et al., 2016). The results of this study demonstrated that the α2,3-type receptor is present in both human and mouse primary tracheal cells. It also showed that the presence of cilia was necessary for infectivity of certain strains of IAV such as rWSN. It also demonstrated species-specific differences in receptor positive cells; mTECs lack α2,6-type receptors and thus are not susceptible to strains of influenza that can only utilize that receptor (Yan et al., 2016). Other studies have been conducted that examined receptor type in hNECs from several individuals the results of which again demonstrated viral preference for certain sialic receptor types (Ibricevic et al., 2006).

Ultimately, it is the receptor type present in the susceptible cells as well as the sialic acid residue preference of the virus that determines how efficiently and effectively a virus can infect a host.

Innate Immune Responses to Influenza Viruses

In humans, once IAV invades the epithelial tissues in the respiratory tract the immune cells recognize the pathogen and begin to respond. Within the mucosal surface, nonspecific proteins try to bind the influenza particle and clear it via the mucocilary apparatus (Mandell et al., 2014). If this is ineffective, then the virus enters the host cell triggering the innate immune response. When IAV first encounters respiratory epithelial cells and penetrates them to begin replication, pattern recognition receptors (PRRs), including the toll-like receptors (TLR) such as TLR3, 7, 8 and 9, within the epithelial tissues recognize pathogen-associated molecular patterns (PAMPS) on the virus. Retinoic acid-inducible gene I (RIG-I) and the NOD-like receptors (NLRs) are also activated to respond to IAV infiltration upon their recognition of viral RNA or DNA (Rouse & Sehrawat, 2010). The activation of RIG-I is essential for the induction of the innate immune response to influenza A virus as it leads to a signaling cascade for the production of pro-inflammatory cytokines, type I interferons, antimicrobial peptides, and chemokines, as well as the recruitment of other inflammatory cells (Short et al., 2014). Importantly, the interferon response is believed to be essential for combating the replication of virus (Gack et al., 2007).

Interferons are induced by infected cells in order to up-regulate anti-viral mechanisms. Infected epithelial cells detect influenza A virus via the RIG-I/MAVS PRR. Viral detection leads to the activation of IRF7 (Crotta et al., 2013) and the induction of type I and III interferons, including IFN-α, IFN-β, and IFN-λ (Sommereyns et al., 2008). Type I IFNs, such as IFN-α and IFN-β are essential in the innate immune response to influenza infection (Muller et al., 1994); they signal through the JAK-STAT pathway to induce various interferon-stimulated genes such as protein kinase R (PKR) and 2′–5′ oligoadenylate synthetases (OAS) (Stark et al., 1998) the products of which go on to limit virus replication via the inhibition of host cellular translation, degredation of host and viral RNA, and disruption of the viral polymerase complex (Garcia-Sastre & Biron, 2006). While type 1 IFNs are expressed on all cell types, type III IFNs are primarily found on epithelial cells in the gastrointestinal and respiratory tract indicating they may act in a site-specific manner to induce innate immune responses to influenza A virus within epithelial tissue (Crotta et al., 2013). This site specificity has been demonstrated in mice following viral infection where it was found that type I IFNs were robustly expressed in the brain following infection but type III expression was low and restricted to only epithelial cells at that site (Sommereyns et al., 2008). Unfortunately, the influenza virus has several mechanisms for manipulating host immune response and gene expression in order to dampen the interferon response.

Influenza A virus limits type I IFN production by interfering with RIG-1 signaling. Viral NS1 protein prevents the translocation of IFR-3 and NF-κB (Wang et al., 2000), this prevents the transcription of type I IFNs from occurring (Randall & Goodbourn, 2008).

By preventing IFN synthesis, influenza A virus can continue to replicate in host cells. The NS1 protein has also been shown to down regulate general host gene expression mainly through its interaction with host zinc finger domains on the nuclear cleavage and polyadenylation specificity factor (CPSF30) (Nemeroff et al., 1998). CPSF30 is necessary for processing the 3' end of cellular pre-mRNA, without it RNA synthesis cannot occur (Nemeroff et al., 1998). NS1 complexes with CPSF30 blocking the production of mature, polyadenylated host mRNAs (Fores et al., 1994). Host cells may try to respond to this disruption in host cell activities by inducing apoptosis; however, it has been demonstrated that influenza virus infected cells may be able to evade apoptosis due to down-regulation of type I IFNs (Zhirnov et al., 2002) by NS1. This is advantageous to the virus as it allows continued replication without being recognized and destroyed by the host immune system.

While it is believed that interferons are important for the innate immune response to virus, recent research has shown that other innate immune factors may activated by a novel pathway independent of and occurring before the interferons within the epithelial surface. Researchers infected wild-type C57BL/6 mice intravaginally with HSV-2 and then collected washes to look for viral mRNA as well as innate immune factors such as chemokines and cytokines (Iversen et al., 2016). Type I and III interferons were not detected in the washes until two days post infection. However, CXCL9 and CXCL10 were detected a full 24 hours before any interferon production could be sensed (Iversen et al., 2016). These two chemokines signal through the same receptor, CXCR3, and can be induced by interferons or other proinflammatory signals

(Groom & Luster, 2011). The authors of this study were ultimately able to show that it was the disruption of the mucosa by viral O-linked glycans that induced CXCL10 production leading to the recruitment of neutrophils in order to protect the host (Iversen et al., 2016). Therefore, while interferons are important for response to IAV infection, there are other innate immune factors that act in defense of the host to viral infection.

While the aforementioned immune factors are required for adequate control and elimination of viral infection, too much inflammation can lead to more severe disease. Dendritic cells, natural killer cells, and macrophages are necessary for the initial control of viral replication, but too robust a response can lead to destructive inflammation in the respiratory tract. In humans, non-human primates, and mice this has been demonstrated by examining lung pathology from individuals who succumbed to H5N1. Retrospective data from the 1918 H1N1 pandemic was also instrumental in demonstrating this observation (Peiris et al., 2009). Examination of both events reveals that cytokine and chemokine-induced inflammation in the lungs of infected individuals leads to respiratory distress that proved fatal in those infections (Kash et al., 2006).

Influenza Epidemiology

Influenza viruses cause disease in one-tenth of the adult population of the world per year mostly through seasonal epidemics and, less frequently, through avian influenza outbreaks and pandemics (WHO, 2015). The very young, the very old,

pregnant women, and individuals with compromised immune systems are at the highest risk of complications arising from influenza infection. Those who suffer from complications such as secondary bacterial infections are at increased risk of mortality, and, on average, between two and five hundred thousand people worldwide die from influenza per year (WHO, 2015).

The sex of the infected individual may impact disease severity. Women of reproductive ages (i.e., between the ages of 18 and 45), experience greater morbidity and mortality when compared to age-matched men during epidemic, pandemic, and localized outbreaks. Case morbidity and mortality data from the H5N1 avian influenza outbreak in Asia demonstrates that women between the ages of ten to thirty-nine experience worse outcomes when compared to their male counterparts (Rabinowitz et al., 2010). Similarly, data from the initial wave of the 2009 H1N1 pandemic also show that women between the ages of 18 and 65 have increased incidence and severity of disease when compared to similar aged men (Klein et al., 2010). Most recently, the incidence of H7N9 avian influenza in China also shows that females of reproductive age are more likely than males to die from H7N9 (Bridges et al., 2003). In general, after puberty and prior to menopause, women experience worse influenza outcomes than age-matched men (Klein et al., 2010).

The causes of increased susceptibility to influenza in women of reproductive ages are diverse and including both sex and gender associated variables. Sex refers to a person's biological composition i.e. male, female or intersex. Gender refers to how a culture feels and behaves in reference to that person's biological sex (Practice

Guidelines, 2011). Gender-associated factors, such as occupation, access and utilization of health care services, and hygiene may contribute to differential outcome from influenza between the sexes. In many countries, health workers, nurses, and primary school teachers are predominantly women. Women are also the primary care givers to young children in both developing and developed nations (Bridges et al., 2003). Healthcare patients and children in schools cluster, making disease transmission more likely, consequently those in closest contact are also at greatest risk for exposure to seasonal influenza viruses. In contrast, despite women being more likely to be hospitalized following exposure to avian influenza viruses (Fasina et al., 2015), men and women are at equal risk for exposure to avian influenza is via their contact with poultry. On larger farms or in commercial animal facilities, males make up the majority of the work force while women tend to homestead operations and smaller farms (Jagger et al., 2012). Whether biological differences between men and women, including circulating sex steroids, contribute to differential risk of severe influenza is rarely considered in epidemiological and clinical studies.

Sex-Specific Differences in Influenza

Inflammation

Male-female differences in infection may be caused by biological differences between men and women, including effects of endogenous sex steroid hormones on cellular function. Mice have been used to examine and model these differences. Using

murine adapted H1N1 and H3N2 in C57BL/6 mice, it was shown that female mice have lower lethal dose$_{50}$ (LD_{50}) levels when compared to their mass-equivalent male counterparts (Lorenzo et al., 2011). Female mice also had higher levels of neutralizing and total antibodies against both inoculation and primary infection than male mice (Lorenzo et al., 2011). Another study demonstrated that in adult male and female mice infected with a lethal dose of a mouse adapted H1N1 (A/Puerto Rico/8/1934; PR8), mortality was increased in females compared to males (Robinson et al., 2011). Female mice began dying sooner than their male counterparts and experienced significantly more mortality over the course of the study (Robinson et al., 2011). Innate immune responses contribute to these differences seen between male and female mice. It has been demonstrated that PRRs from female mice, such as the TLRs, are more adept at detecting viral nucleic acids than male PRRs (Klein et al., 2010). It has also been shown that while IAV titers in the lungs of male and female mice are the same (Robinson et al., 2011), the overall levels of pro-inflammatory cytokines and chemokines such as CCL2, TNF-α, IFN-γ and IL-6 are greater in female mice than males (Larcombe et al., 2011). A difference in cytokine production, not viral titer, may contribute to the increased morbidity and mortality in female compared to male mice.

Sex steroid hormones

Endogenous sex steroid hormones have effects on the outcome of influenza infection. In female mice infected with H1N1, the estrous cycle is interrupted resulting in low circulating levels of estrogen and progesterone. This leads to an increase in NF-κB transcription and resulting excessive expression of proinflammatory chemokines and

cytokines (Robinson et al., 2011). By administering estrogen to ovarectomized female mice during infection, pro-inflammatory chemokines and cytokines were suppressed increasing survival of the infected female mice (Robinson et al., 2011). In mice, estrogen exerted its effects through the ER-α receptor, which is found on dendritic cells and macrophages, by suppressing NF-κB activity (Robinson et al., 2011).

In males, testosterone exerts effects on cells of the immune system. In cell culture it has been demonstrated that testosterone is anti-inflammatory, inhibiting the production of TNF-α and nitric oxide while simultaneously inducing IL-10 (D'Agostino et al., 1999). The effect of testosterone on macrophages has also been examined in mice. It was demonstrated that in vitro exposure of murine macrophages to testosterone decreased the cell surface expression of Toll-like receptor 4, a necessary PRR for activation of the pro-inflammatory NF-κB pathway. This pathway is necessary for the transcription of interferon producing genes a necessary factor in combating IAV infection (Rettew et al., 2008).

Sex chromosomes

Finally, in addition to gonadal hormones exerting a direct effect on immune effects, it has been proposed that genes found on the X and Y-chromosomes may impact sex-related differences in influenza pathogenesis. X-chromosome linked DEAD-box helicase 3 (DDX3X) is a helicase that is necessary for the production of interferon (Soulat et al., 2008). Females have two copies of DDX3X while males have one copy of DDX3X and one copy of the Y-chromosome linked homologue. Studies have demonstrated that expression levels of homologous sex linked genes vary (Wilson &

Makova, 2009); females having two copies of DDX3X may therefore have increased levels of this helicase and subsequently greater IFN production (Muller et al., 2013).

Studies on sex chromosomes and influenza A virus infection in four core genotype (FCG) mice have also demonstrated how sex steroids rather than the sex chromosome complement may mediate disease pathogenesis (Robinson et al., 2011). FCG mice have the Sry gene, a gene on the Y chromosome that is responsible for initiating testes formation and testosterone synthesis (Koopman et al., 1991), deleted and a Sry transgene inserted onto an autosome (Robinson et al., 2011). Infection with IAV in wild type gonadally intact females resulted in higher morbidity and mortality when compared to wild type gonadally intact males. This observed sex difference in gonadally intact mice disappeared when both sexes of mouse were gonadectomized (Robinson et al., 2011). FCG mice were gonadectomized and then infected with IAV; there was no observed difference in morbidity or mortality between the male and female mice. This result indicates that in this model sex hormones, not sex chromosomes, are responsible for the observed difference in IAV pathogenesis (Robinson et al., 2011).

Aims and hypotheses

While immortalized cell lines and mouse models have been used to examine the effects of sex on inflammation and infection, there has been little work done in humans. As a surrogate to infecting humans, primary human epithelial cell cultures can be used

to study the dynamics of influenza infection and host innate immune responses. In my project, I investigated differences in viral replication and the host innate immune responses following IAV infection in male and female differentiated human nasal epithelial cell (hNEC) cultures. Nasal cells are the first site of IAV infection, making them an excellent model to study IAV entry and early IAV replication kinetics (Muller et al., 2013). Previous work in hNECs characterized viral replication kinetics and proteins of the host cell following infection with live attenuated influenza virus (LAIV) as well as wild type strains (Fischer et al., 2015). It was demonstrated that there are replication kinetic differences of both LAIV and wild type in hNECs versus the immortal MDCK cell line. Furthermore, there were differences in M2 protein production in hNECs infected with LAIV versus the MDCK counterpart (Fischer et al., 2015). The use of hNECs over nonhuman MDCK cells allows us to better understand the human response to IAV infection and the innate immune effectors associated with influenza pathology. Furthermore, by looking at hNECs from both male and female donors sex comparisons of viral replication kinetics, cytokine and chemokine response, and other innate immune factors can be examined.

METHODS

MDCK cells

Madin-Darby canine kidney (MDCK) cells were propagated in Dulbecco's modified Eagle's low-phenol, high glucose medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; Life Technologies), 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies), and 2mM L-Glutamine (Life Technologies). The cells were maintained at 37C in a humidified environment with 5% CO2.

Virus

The recombinant influenza A/Udorn/307/72 H3N2 virus used in this study was described previously (Pekosz et al., 2009). The hemagglutinin (HA) protein contains amino acid changes (HA L226Q/S228G in the H3 numbering system) that allows for preferential binding to α2,3-linked sialic acid. Viral working stocks of rUdorn α2,3 were generated by infecting MDCK cells at a multiplicity of infection (MOI) of approximately 0.01 infectious units per cell in low-phenol, high glucose DMEM containing 0.25% bovine serum albumin (BSA; Life Technologies), acetylated trypsin from bovine pancreas (5µg/mL; N-acetyl trypsin; Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 6mM L-Glutamine, and 1mM Sodium Pyruvate (Life Technologies), which we refer to as infection media. The infected cells were incubated at 37°C for 72 hours, infected cell supernatant was harvested, clarified by centrifugation at 500g for 10 minutes, aliquoted

and stored at -80C. Infectious virus titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) as described previously (McCown & Pekosz, 2005).

Human nasal epithelial cell (hNEC) cultures

Primary human nasal epithelial cells were obtained from non-diseased human donor tissue during endoscopic sinus surgery. The cells were differentiated at an air-liquid interface (ALI) in 24-well Falcon filter inserts (0.4-µM pore; 0.33cm^2; Becton Dickinson) coated with human type IV placental collagen (Sigma-Aldrich) as described previously (Fischer et al., 2015; Kohanski & Lane, 2015; Bajic et al., 2003).

Infection and treatment of hNEC cultures

Prior to infection, the apical surface of hNEC cultures was washed with Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS; Life Technologies). The cultures were infected via the apical chamber with a multiplicity of infection (MOI) of 1.0, 0.1, or 0.01 $TCID_{50}$/cell or mock infected at 32°C in 200 ul of infection media. After 2 hours, the inoculums were aspirated and the apical surfaces washed twice with DPBS before returning the cells to incubate at 32°C. At the indicated times post infection, 200 ul of infection media was added to the apical surface, incubated at 32°C for five minutes, then collected. All samples were stored at -80°C until subjected to measurement of infectious viral production via $TCID_{50}$ assay using MDCK cells or measurement of host immune factors via multiplex array analyses (Meso

Scale Discovery) and enzyme linked immunosorbent assays (ELISA). Basolateral DMEM was collected and replaced with media containing fresh vehicle or compound at 48 and 96hpi.

Tissue Culture Infectious Dose ($TCID_{50}$) assay

MDCK cells were plated in a 96-well plate and cultured for 72 hours in growth DMEM until 90-100% confluent. The cells were washed twice with DPBS, then covered with 180µL of infection DMEM. Serial dilutions (10^{-1} to 10^{-8}) of hNEC apical samples at each time-point were made in separate, 96-well plates by adding 20µL of each apical sample to 180µL of infection DMEM, then serially diluted 10 fold. Twenty µL of each dilution was then added to the MDCK plates in replicates of six, resulting in final dilutions of each sample ranging from 10^{-2} to 10^{-9}. The infection proceeded for 7 days at 32°C, then the cells fixed with 4% formaldehyde and stained with napthol blue black solution (napthol blue black 1.6mM; sodium acetate 165mM; glacial acetic acid 60mL; qs to 1 liter). The cytopathic effect was scored visually and the Reed and Muench calculation was used to determine the titer of infectious virus at each time-point (McCown & Pekosz, 2005).

Multiplex Assay Analysis

The Meso Scale Discovery (MSD, Gaithersburg, MD) multiplex assay system was used to measure chemokines collected from apical and basolateral samples of hNECs

after infection. In each well, the Chemokine Panel 1 kit quantitatively determined the concentration of eight C-C ligand motif (CCL2, CCL3, CCL4, CCL11, CCL13, CCL17, CCL22, CCL26) and two C-X-C ligand motif (CXCL8, CXCL10) chemokines, according to the manufacturer's protocols. All samples were run in duplicate. MSD plates were read on the MSD SECTOR Imager 2400 at the Becton Dickinson Core Facility at the Johns Hopkins Bloomberg School of Public Health and analyzed on the accompanying software (MSD Discovery Workbench version 4).

ELISAs

The PBL Assay Science (Piscataway, NJ) DIY Human IFN Lambda 1/2/3 (IL-29/28A/28B) was used to measure levels of interferon lambda (IFNλ) collected from apical and basolateral samples of hNECs after infection. All samples were run in duplicate and read on the FilterMax F3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). The data were analyzed with the accompanying software (SoftMax Pro Data Acquisition and Analysis Software).

Microarray Analyses

The microarray analyses were done using the Genomic Analysis and Sequencing Service Center Core Facility within the W. Harry Feinstone Department of Molecular Microbiology and Immunology. Trizol Reagent and the PureLink RNA Mini kit (Ambion/Life Technologies) were used for extraction and purification of RNA. Cells were

processed according to the manufacturer's protocol including the on-column DNAse treatment. Following elution of purified RNA from the PureLink columns with Nuclease-free water, quantitation was performed using a NanoDrop spectrophotometer and quality assessment determined by RNA Nano LabChip analysis on an Agilent BioAnalyzer 2100 or RNA Screen Tape on an Agilent TapeStation 2200. One hundred nanograms of total RNA was processed for hybridization to Affymetrix Human Gene ST 2.0 microarrays using the Affymetrix GeneChip WT PLUS Reagent Kit according to the manufacturer's recommended protocol. Hybridization was performed at 45^{o}C for 16 hours at 60 rpm in the Affymetrix rotisserie hybridization oven. The signal amplification protocol for washing and staining of eukaryotic targets was performed in an automated fluidics station (Affymetrix FS450) using Affymetrix protocol FS450_0002. The arrays were scanned in the GCS3000 laser scanner with autoloader and 3G upgrade (Affymetrix). Quality assessment of hybridizations and scans was performed with Expression Console software (Affymetrix).

Statistical Analyses

Data were expressed as means +/- standard error of the mean (SEM), and multiple comparisons between experimental groups were made using multivariate analysis of variance (MANOVA) and/or analysis of variance (ANOVA) followed by Tukey's post hoc comparisons, when appropriate. For microarray analyses, Partek Genomics Suite version 6.6 was used for detailed statistical analysis. The RMA (Robust Multi-chip

Average) Algorithm was used for background correction, normalization, and summarization of probes. Analysis of Variance (ANOVA) with linear contrasts was performed to generate p-values and fold changes; numerous plots (PCA, Volcano, Interaction, Dot, etc); and creation and querying of gene lists. At least three separate experiments were conducted for each treatment prior to data analysis. Statistical significance was assigned at $p \leq 0.05$.

RESULTS

rUdornα2,3 replication kinetics are not different in hNECs derived from male and female donors

To test the hypothesis that viral replication kinetics might differ depending on the sex of the donor cell, primary human nasal epithelial cell (hNEC) cultures from male and female donors of reproductive age were infected with A/Udorn/307/72 H3N2 α2,3 (rUdorn α2,3) at an MOI of 0.1. Apical supernatant samples were taken every 24 hours for 5 days to measure virus titers (Fig. 1). Peak virus replication occurred at roughly 72 hours post infection in hNECs from both male and female donors. Both the replication kinetics and peak virus titers were similar in hNECs derived from male and female donors. These data indicate that following rUdorn α2,3 infection, replication kinetics in hNECs do not differ between the sexes. Because sex differences in the outcome of influenza are reported in humans (Klein et al., 2010; Rabinowitz et al., 2010) and animals (Robinson et al., 2011), we hypothesize that sex differences in the outcome of infection may be the result of differences in the host response to infection.

Interferon-λ production is greater in hNECs from females compared to males

Interferon- λ is the primary IFN produced by respiratory epithelial cells in response to infection with IAVs (Crotta et al., 2013). To test the hypothesis that primary

hNEC cultures produce IFN- λ following infection with a H3N2 virus that preferentially binds α2,3, hNECs from female and male donors were infected with rUdorn α2,3 at an MOI of 0.1 and apical and basolateral supernatant samples were taken every 24 and 48 hours respectively following infection. Detectable interferon-λ production was observed at 96 hours post infection in both apical (Fig. 2A) and basolateral samples (Fig. 2B). Both apical and basolateral samples from rUdorn α2,3-infected cells, regardless of sex, produced more IFN- λ than the mock-infected samples. Overall, basolateral production of IFN-λ was greater than apical IFN-λ production regardless of the sex of the hNECs donor. In the apical supernatants (Fig. 2A), hNECs from females produced significantly more IFN-λ when compared to cells from their male counterparts. This sex difference in production was not seen in the basolateral samples (Fig. 2B). Taken together, these data suggest that there is increased production of apical IFN-λ in hNECs from female donors.

Chemokine production following rUdorn α2,3 infection is more robust in hNECs from male donors than female donors

During IAV infections, chemokines are produced by epithelial cells to signal immune cells to the site of infection (Iversen et al., 2016; Groom & Luster, 2011). To test the hypothesis that chemokine production might differ in cells derived from male or female donors, we examined chemokine production in apical samples from hNEC cultures at 48, 72, and 96 hours post infection. The chemokine panel used for the analysis contained the following ten chemokines: CCL11, CCL26, CCL2, CCL13, CCL22,

CCL3, CCL4, CCL17, CXCL8, and CXCL10. We first compared rUdorn α2,3-infected to mock-infected hNEC cultures within each sex. Of the ten chemokines examined, rUdorn α2,3-infected hNECs from females produced all but CCL26, CXCL8, CCL2, and CCL13 at levels significantly greater than mock (Table 1A) while males produced all but CCL26 and CCL13 at levels significantly greater than mock (Table 1B). These data suggest that within each sex there is an increase in chemokine production in response to rUdorn α2,3 infection in hNECs from derived from both male and female donors, but possibly to a greater extent in cultures from males.

To test the hypothesis that there is differential expression of chemokines between the sexes, the expression levels of each chemokine examined (Tables 1A, 1B) from rUdorn α2,3 –infected cultures was normalized to same sex mock-infected cultures and then compared between the sexes. After normalization, hNECs from male donors produced significantly more CXCL10, CCL17, CCL2, and CCL22 at the various time points post-infecgtion than hNECs from female donors. These four chemokines are important for the IFN response to infection as well as the recruitment of immune cells in response to infection (De Paepe et al., 2009). In contrast, the only chemokine that was produced at higher concentrations in hNECs from female donors as compared to males was CCL11, which is generally associated with allergic type responses (Graziano et al., 1999). These data demonstrate that males produce a more robust and varied chemokine response to IAV infection when compared to hNECs from female donors. Furthermore,

the function of the chemokines released in response to IAV infection may also differ between the sexes.

Transcriptional activity in hNECs during rUdorn α2,3 infection is greater in cultures derived from male than female donors

To more fully characterize sex differences in host cellular response to rUdorn α2,3 infection, we conducted global transcriptional analyses using microarrays. For the initial analysis, the RMA Algorithm was used for background correction, normalization, and summarization of probes. An ANOVA with linear contrasts was then performed to genes that were differentially expressed either 24 or 48 hours post-infection. At 24 hours post infection (Figure 4A), only 15 of the roughly 35,000 genes probed on the microarray were differentially expressed between rUdorn α2,3-infected hNECs from male and female donors. In contrast, at 48 hours post infection (Table 2B) there were 826 differentially expressed genes following rUdorn α2,3 infection in hNECs from male and female donors. Furthermore, there were 451 and 578 differentially expressed genes in IAV infected hNECs compared to mock-infected cultures derived from male and female samples respectively (Figure 4B).

For the second analysis, RMA was performed and then rUdorn α2,3-infected cultures were normalized to same sex mock-infected cultures to control for baseline differences between the sexes prior to infection. Following normalization, an ANOVA

was performed in order to detect genes that were differentially expressed between the sexes during infection. Following this analysis, only 16 genes (Figure 4B) were differentially expressed 24 hours post infection between hNECs from male and female donors. By 48 hours post infection, 780 genes were differentially expressed between the sexes (Figure 4B). The same intrinsic sex differences in gene expression following infection were also observed in the normalized data at 48 hours post infection (Figure 4B). Furthermore, there were 492 differentially expressed genes in both normalized and non-normalized data sets at 48 hours post infection (Figure 4C). These data indicate that the transcriptional responses of hNECs from male and female donors differ in a time-dependent manner following rUdorn α2,3 infection.

Transcriptional activity along pathways associated with immune system processes is higher in hNEC cultures derived from males than females

Because we observed the greatest number of differentially expressed genes 48 hours post-infection, we utilized data from this time point and performed a Gene-Ontology (GO)-enrichment to determine the biologically relevant pathways that were differentially activated between the sexes following rUdorn α2,3 infection. An enrichment score of ≥ 3 is considered statistically significant with a greater score indicating a stronger association of the gene set to that biological function. Regardless of whether data were not normalized to same-sex mock-infected cultures (Figure 5A) or were normalized to same-sex mock-infected cultures (Figure 5B) the top three

biologically relevant pathways that showed significant activation following infection included immune system process, response to stimulus, and multi-organism process. Along each of these pathways transcriptional activity was generally higher in hNECs derived from male than female donors. Using hierarchal clustering, which is an unbiased method for assessing the relationships between individual gene expression patterns and discerning how genes group together independent of sex or infection status, we demonstrated (Figure 5C) that gene expression patterns cluster by both sex and infection status. These data indicate that hNECs from male donors have greater differential gene expression of genes associated with these three biological functions when compared to hNECs from female donors.

Within the immune system process genes that were differentially expressed between the sexes 48 hours post-infection, there were 137 common genes that were differentially expressed between the sexes when expression data either were or were not normalized to same sex mock-infected cultures. A majority of these 137 genes were associated with pattern recognition receptor activity, IFN signaling, and inflammatory responses (Fig 6). At 48 hours post rUdorn α2,3 infection, hNECs from male donors had a greater proportion of upregulated immune response genes than their female counterparts. Of the genes that were upregulated in hNEC cultures from males, these genes were generally associated with antiviral responses to IAV infection including IRF7, DDX58, and CXCL10 (Rouse & Sehrawat, 2010; Crotta et al., 2013; Iversen et al., 2016). In contrast, the few genes with higher levels of expression in cultures derived from

females included KIT, CD36, and KIF2C, none of which are known to be involved in the innate immune response to IAV (Roberts & Govender, 2015; Smith et al., 2012; Wang et al., 2015). Taken together, these data suggest that activity along antiviral pathways are upregulated to a greater extent in cultures derived from males during rUdorn α2,3 infection.

Figure 1

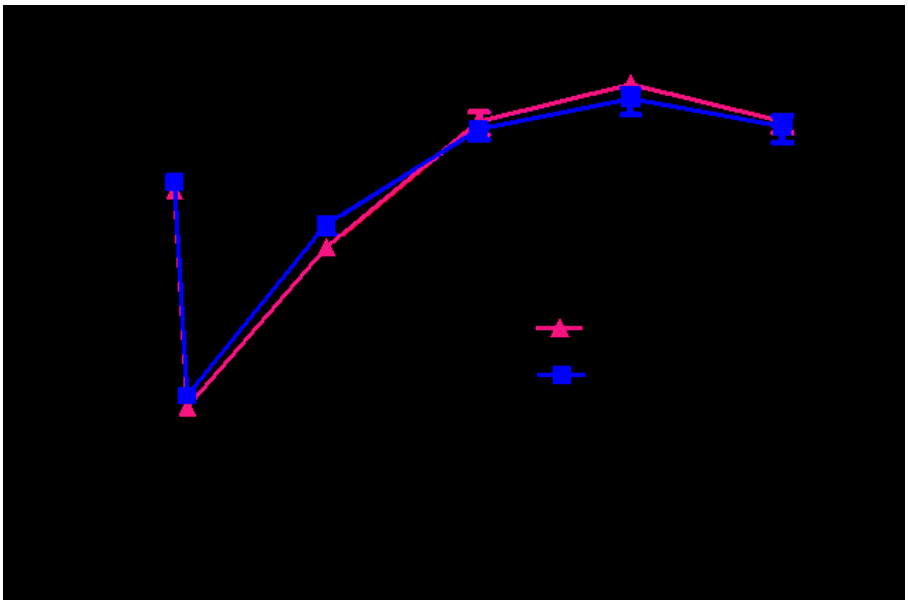


Figure 1: Effect of sex on the outcome of recombinant influenza A/Udorn/307/72 H3N2 rUdorn α2,3 infection in hNECs from female and male donors. hNECs from donors between the ages of 16-45 were infected with rUdorn α2,3 (MOI = 0.1 $TCID_{50}$/cell) via the apical membrane. Apical samples were obtained 2, 24, 48, 72, and 96 hours post infection (n=13 female donors, n=10 male donors). Viral titers were analyzed at these time points by $TCID_{50}$. Data shown are the mean ± SEM. The dashed line represents the limit of detection by $TCID_{50}$. There was no difference in viral replication kinetics or titer between samples from male and female donors.

Figure 2

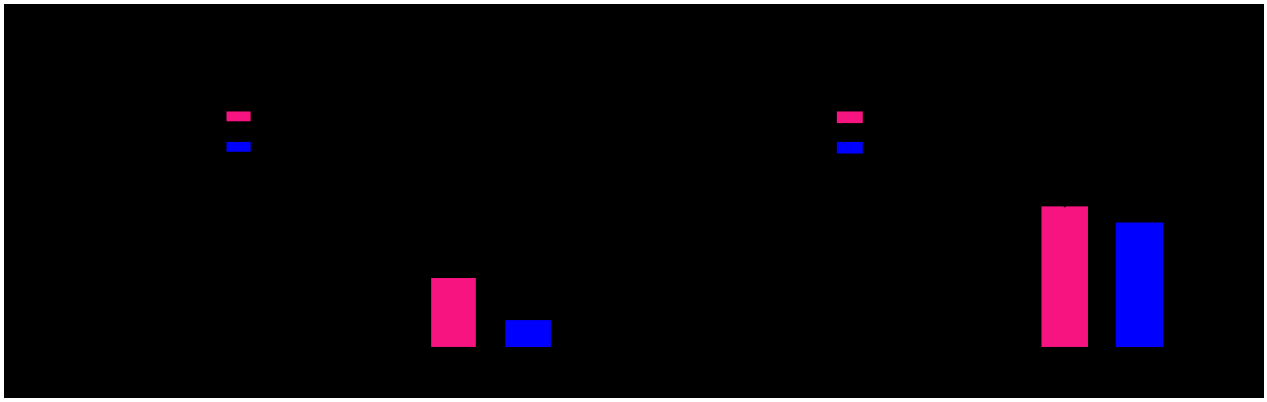


Figure 2: Effect of sex on apical and basolateral interferon lambda (IFN-λ) production. hNECs from male and female donors were infected with rUdorn α2,3 (MOI = 0.1 TCID50/cell) or mock-infected via the apical membrane. Apical (A) and basolateral (B) samples were examined for IFN-λ production 96 hours post infection (n=5 donors per sex). Data shown are the mean ± SEM. *$p \leq 0.01$. hNECs from female donors produced statistically significantly more IFN-λ apically than their male counterpart while there was no sex difference in the basolateral production of IFN-λ. Both sexes produced more IFN-λ basolaterally than apically.

Table 1: rUdorn α2,3-induced chemokines in hNECs from male and female donors 48, 72, and 96 hours post infection. hNECs from male and female donors were infected with rUdorn α2,3 or mock-infected (MOI = 0.1 TCID50/cell) via the apical membrane. Apical samples from female (A) and male (B) hNECs were examined for chemokine production (n=5 donors per sex) at 48, 72, and 96 hours post infection. Data shown are the mean ± SEM. Bolded values denote $p<0.05$ for samples from infected compared with mock-infected cultures. There is differential production of several chemokines following rUdorn α2,3 infection with hNECs from males showing greater production of chemokines compared with cultures from females.

A.

	MOCK (pg/ml)	48hpi (pg/ml)	72hpi (pg/ml)	96hpi (pg/ml)
CCL11 (eotaxin)	3.08 ± 0.50	**45.02 ± 8.51**	**45.49 ± 13.72**	**31.89 ±10.36**
CCL26 (eotaxin-3)	6.25 ± 1.10	7.23 ± 3.00	20.10 ± 10.40	9.35 ± 2.09
CXCL8 (IL-8)	7839.02 ± 2435.02	13242.17 ± 3703.36	17434.01 ± 2332.05	14901.70 ± 3778.89
CXCL10 (IP-10)	35.14 ± 11.46	**2578.00 ± 23.57**	**2578.00 ± 23.57**	**1590.91 ± 346.67**
CCL2 (MCP-1)	21.15 ± 5.89	36.66 ± 10.67	30.89 ± 7.82	17.82 ± 5.64
CCL13 (MCP-4)	5.45 ± 1.93	3.48 ± 1.31	6.29 ± 3.91	1.96 ± 0.32
CCL22 (MDC)	82.46 ± 25.19	129.28 ± 22.94	**190.38 ± 48.09**	**163.44 ± 47.01**
CCL3 (MIP-1a)	12.44 ± 5.25	30.49 ± 3.84	**58.48 ± 10.65**	**37.88 ± 8.44**
CCL4 (MIP-1b)	2.30 ± 0.87	**15.28 ± 2.94**	**22.69 ± 5.27**	**10.70 ± 3.15**
CCL17 (TARC)	9.47 ± 3.93	**25.27 ± 3.86**	**36. 30 ± 8.32**	23.98 ± 10.21

B.

	MOCK (pg/ml)	48hpi (pg/ml)	72hpi (pg/ml)	96hpi (pg/ml)
CCL11 (eotaxin)	6.09 ± 1.68	**34.94 ± 6.03**	**40.09 ± 6.10**	**51.57 ± 7.81**
CCL26 (eotaxin-3)	8.05 ± 3.38	23. 87 ± 14.77	20.20 ± 8.40	14.03 ± 4.88
CXCL8 (IL-8)	2135.80 ± 865.90	2243.15 ± 678.11	**14952.26 ± 6814.14**	**19339.96 ± 6435.76**
CXCL10 (IP-10)	11.97 ± 2.98	**2014.54 ±268.86**	**2499.07 ± 82.06**	**2578.00 ± 23.57**
CCL2 (MCP-1)	8.42 ± 3.01	**76.76 ± 28.72**	51.00 ± 17.51	29.75 ± 11.03
CCL13 (MCP-4)	4.09 ± 1.31	7.87 ± 4.34	4.90 ± 2.30	7.49 ± 4.02
CCL22 (MDC)	30.57 ± 8.87	74.89 ± 12.60	**124.03 ± 17.33**	**197.62 ± 24.71**
CCL3 (MIP-1a)	13.20 ± 4.84	36.65 ± 6.64	**62.95 ± 14.98**	**62.48 ± 10.77**
CCL4 (MIP-1b)	3.69 ± 1.69	9.68 ± 1.59	**17.62 ± 2.96**	**18.02 ± 2.26**
CCL17 (TARC)	5.94 ± 1.61	18.17 ± 3.43	19.46 ± 3.13	**31.47 ± 3.80**

Figure 3

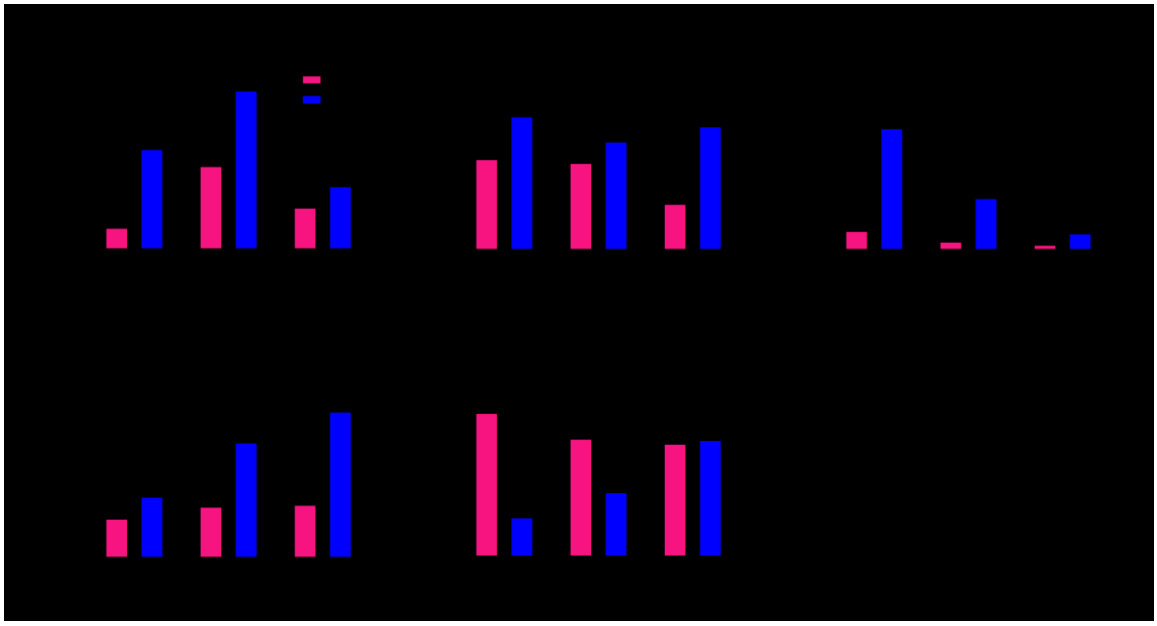


Figure 3: Effect of sex on chemokines production in response to rUdorn α2,3 infection at various time points post-infection. The concentration of chemokines from infected hNECs derived from males or females (Table 1) were normalized to their respective mock-infected cultures and then compared between the sexes at each of the time points (n=5 donors per sex). Data shown are the mean ± SEM. Asterisks (*) denote $p<0.05$. hNECs from male donors produced statistically significantly more CXCL10, CCL17, CCL22, and CCL2 than their female counterparts. CCL11 was the only chemokine that was produced in higher concentrations in hNECs from female than male donors.

Figure 4

A.

24 hours post-infection	FC ±	p-value	DE Genes
Male Virus v. Mock	2	pFDR <0.05	4
Female Virus v. Mock	2	pFDR <0.05	0
Male Virus v. Female Virus	2	pFDR <0.05	15
48 hours post-infection			
Male Virus v. Mock	2	pFDR <0.05	451
Female Virus v. Mock	2	pFDR <0.05	578
Male Virus v. Female Virus	2	pFDR <0.05	826

B.

24 hours post-infection	FC ±	p-value	DE Genes
Male Virus v. Mock	2	pFDR <0.05	0
Female Virus v. Mock	2	pFDR <0.05	0
Male Virus v. Female Virus	2	pFDR <0.05	16
48 hours post-infection			
Male Virus v. Mock	2	pFDR <0.05	445
Female Virus v. Mock	2	pFDR <0.05	405
Male Virus v. Female Virus	2	pFDR <0.05	780

C.

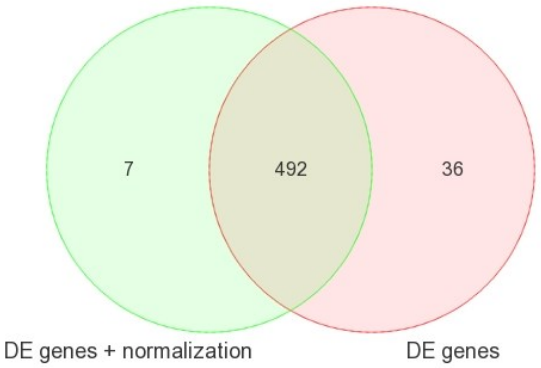
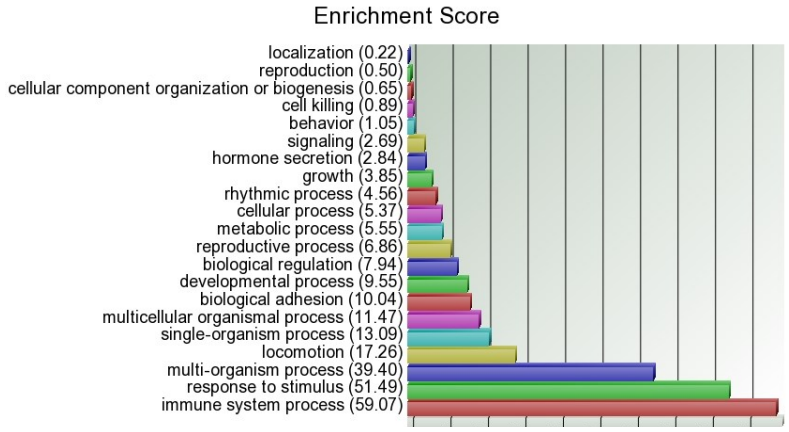


Figure 4: Differentially expressed genes in cultures 24 and 48 hours rUdorn α2,3-infection. hNEC cultures from male and female donors were infected with IAV (MOI = 0.1 TCID50/cell) via the apical membrane. At 24 and 48hpi, cells were harvested and

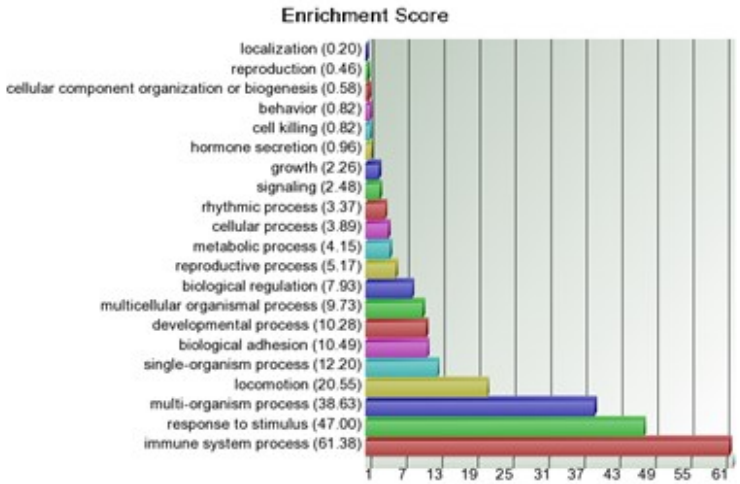
RNA isolated and spotted onto Affymetric Human GeneST2.0 arrays. The number of differentially expressed genes were calculated based on RMA normalization only (A) or RMA normalization following normalization of infected cultures to same-sex mock-infected cultures (B). Using either analysis, there were a limited number of differentially expressed genes at 24 hours post infection and a much greater number of differentially expressed genes 48 hours post infection. Those differentially expressed genes were present in both normalized and non-normalized data sets (C).

Figure 5

A.


Enrichment Score
localization (0.22)
reproduction (0.50)
cellular component organization or biogenesis (0.65)
cell killing (0.89)
behavior (1.05)
signaling (2.69)
hormone secretion (2.84)
growth (3.85)
rhythmic process (4.56)
cellular process (5.37)
metabolic process (5.55)
reproductive process (6.86)
biological regulation (7.94)
developmental process (9.55)
biological adhesion (10.04)
multicellular organismal process (11.47)
single-organism process (13.09)
locomotion (17.26)
multi-organism process (39.40)
response to stimulus (51.49)
immune system process (59.07)

B.


Enrichment Score
localization (0.20)
reproduction (0.46)
cellular component organization or biogenesis (0.58)
behavior (0.82)
cell killing (0.82)
hormone secretion (0.96)
growth (2.26)
signaling (2.48)
rhythmic process (3.37)
cellular process (3.89)
metabolic process (4.15)
reproductive process (5.17)
biological regulation (7.93)
multicellular organismal process (9.73)
developmental process (10.28)
biological adhesion (10.49)
single-organism process (12.20)
locomotion (20.55)
multi-organism process (38.63)
response to stimulus (47.00)
immune system process (61.38)
1 7 13 19 25 31 37 43 49 55 61

C.

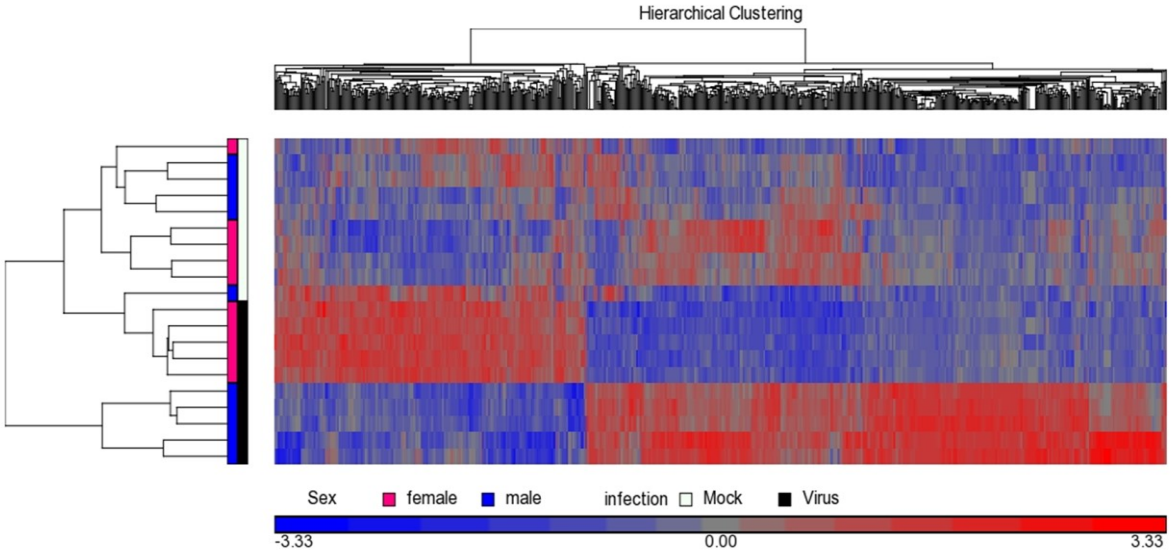

Hierarchical Clustering
Sex female male
infection Mock Virus
-3.33
0.00
3.33

Figure 5: GO enrichment and hierarchical clustering of genes differentially expressed in cultures from male and female donors 48 hours post rUdorn α2,3-infection. hNEC cultures from male and female donors were infected with rUdorn α2,3 (MOI = 0.1 TCID50/cell) via the apical membrane. At 24 and 48hpi, cells were harvested and RNA isolated and spotted onto Affymetric Human GeneST2.0 arrays. Following identification of differentially expressed genes via ANOVAs in Partek Genomic Suit version 6.0, a GO-enrichment was performed on RMA normalized (A) and data normalized with RMA and to same-sex mock infected cultures (B). Using GO enrichment, in both sets of data immune system processes, response to stimulus, and multi-organism processes were the top three differentially activated pathways. A heat map (C) was also generated using the list of 137 genes differentially expressed between the sexes 48 housr post rUdorn α2,3-infection to evaluate the unbiased clustering of these genes which clearly clustered based on the sex and infection status of the cultures.

Table 2: 137 genes that were differentially expressed between the sexes 48 hours post infection. Using the results of the GO-enrichment of RMA normalized and data normalized with RMA and to same-sex mock infected cultures, a list of 137 differentially expressed immune system process genes between hNECs from male and females was derived from both data sets. Fold change and ratio values represent the comparison of virus infected hNECs from females 48 hours post infection compared to their male counterparts.

Gene	p-Value	Fold Change (Male vs Female)	Ratio (Male vs Female)
ISG15	4.74E-14	10.9269	1.09E+01
THEMIS2	2.08E-05	2.30106	2.30E+00
TINAGL1	0.000119531	3.81586	3.81586
KIF2C	5.93E-05	-2.06742	4.84E-01
IFI44L	1.73E-08	14.1501	1.42E+01
IKBKE	0.000400793	2.08585	2.08585
IL19	1.18E-09	4.13149	4.13E+00
CD55	5.47E-05	2.06829	2.06829
IFI6	6.21E-08	9.45863	9.46E+00
GBP1	1.08E-09	4.56431	4.56E+00
GBP2	1.70E-13	4.9136	4.91E+00
GBP2	2.17E-09	3.73138	3.73E+00
S100A12	4.96E-05	3.1957	3.20E+00
IFIT2	1.01E-11	17.8527	1.79E+01
IFIT3	2.07E-10	12.4106	1.24E+01
IFIT1	3.86E-10	12.8766	1.29E+01
IFIT5	9.02E-10	3.07857	3.08E+00
KLF6	0.000146291	2.49704	2.49704
IFITM2	1.68E-06	3.11051	3.11E+00
IFITM1	3.98E-08	4.68754	4.69E+00
TRIM22	2.59E-10	3.69981	3.70E+00
SAA1	0.000646168	4.25031	4.25031
BIRC3	1.16E-07	3.98143	3.98E+00
IFITM3	3.20E-09	3.00073	3.00E+00
IRF7	7.35E-14	4.02932	4.03E+00

Gene	p-Value	Fold Change (Male vs Female)	Ratio (Male vs Female)
TRIM21	1.61E-10	2.76544	2.77E+00
TRIM5	2.89E-12	2.7699	2.77E+00
UBE2L6	3.61E-09	2.91627	2.92E+00
MMP1	0.000246888	3.09477	3.09477
POU2AF1	1.90E-07	-2.89443	3.45E-01
ETS1	0.000240145	2.24002	2.24002
IRAK3	9.57E-07	4.54795	4.55E+00
OAS1	1.45E-10	7.74695	7.75E+00
OAS3	5.98E-12	9.76151	9.76E+00
OAS2	1.78E-12	6.33946	6.34E+00
STAT2	1.06E-09	2.88321	2.88E+00
DUSP6	0.000627573	2.22598	2.22598
OASL	3.07E-13	11.0529	1.11E+01
IRF9	5.04E-09	2.24848	2.25E+00
ADSSL1	9.66E-07	-2.61492	3.82E-01
THBS1	1.33E-05	9.42514	9.43E+00
GCNT3	3.97E-05	3.40362	3.40E+00
PML	5.78E-09	3.25678	3.26E+00
ISG20	5.42E-10	2.10193	2.10E+00
IL4R	0.000712604	2.0745	2.0745
CCL22	4.38E-08	2.45199	2.45E+00
CX3CL1	0.00013702	2.48464	2.48464
SLC7A5	0.000278307	5.94704	5.94704
ITGA3	0.000323013	2.00481	2.00481
CXCL16	7.58E-07	2.8758	2.88E+00
CCL5	8.17E-14	3.55677	3.56E+00
DHX58	3.63E-08	2.26435	2.26E+00
TRIM25	9.22E-08	2.46898	2.47E+00
SECTM1	4.01E-05	3.05476	3.05476
PMAIP1	7.63E-05	2.16042	2.16042
ICAM1	2.20E-10	7.27738	7.28E+00
HSH2D	5.07E-11	2.7941	2.79E+00
IFNL2	3.76E-12	10.6675	1.07E+01
IFNL1	1.76E-10	3.05895	3.06E+00
CEACAM6	0.00087449	3.80777	3.80777
SBNO2	1.42E-05	2.14561	2.15E+00
C3	1.97E-05	4.1205	4.12E+00
BST2	2.25E-07	3.44785	3.45E+00
IFNL3	1.30E-07	2.8282	2.83E+00

Gene	p-Value	Fold Change (Male vs Female)	Ratio (Male vs Female)
RSAD2	3.16E-12	14.6518	1.47E+01
IL1R1	5.98E-07	2.58576	2.59E+00
IL36G	0.000814683	3.17868	3.17868
CCL20	1.21E-12	7.38449	7.38E+00
EIF2AK2	5.40E-15	3.08014	3.08E+00
IGKC	0.0019081	3.68353	3.68353
IL1A	1.02E-07	18.3396	1.83E+01
IL1B	3.48E-05	4.76522	4.77E+00
DPP4	0.000101033	2.38703	2.38703
IFIH1	2.62E-14	6.56558	6.57E+00
STAT1	4.35E-12	4.14128	4.14E+00
BPIFA1	0.000220435	-5.23847	0.190895
ZBP1	3.10E-09	2.23504	2.24E+00
MX2	7.17E-09	2.86843	2.87E+00
MX1	4.31E-13	4.55046	4.55E+00
IGLC1	4.96E-12	2.87579	2.88E+00
APOL1	1.93E-11	4.4137	4.41E+00
APOBEC3A	0.000193241	2.04818	2.05E+00
LIF	0.000152276	2.17405	2.17405
IRAK2	3.87E-06	2.41458	2.41E+00
KIF15	0.000111762	-2.42838	4.12E-01
POLQ	0.000169072	-2.48758	0.401996
PLSCR1	5.21E-09	2.58139	2.58E+00
LAMP3	1.10E-16	6.75476	6.75E+00
KIT	1.24E-07	-7.69578	1.30E-01
EREG	3.16E-05	6.1667	6.17E+00
ANXA3	1.87E-06	3.5699	3.57E+00
HERC5	6.45E-11	10.6909	1.07E+01
CXCL5	4.03E-05	11.9589	1.20E+01
CXCL3	0.00053429	2.31011	2.31011
CXCL9	9.80E-11	29.4025	2.94E+01
CXCL10	9.44E-12	127.751	1.28E+02
CXCL11	9.54E-15	48.304	4.83E+01
SNCA	7.77E-09	-2.72397	3.67E-01
TNIP3	3.72E-08	2.99741	3.00E+00
DDX60	1.03E-10	5.36791	5.37E+00
SPEF2	0.000227991	-2.59144	0.385886
CYFIP2	2.44E-06	-2.58712	3.87E-01
FYB	1.75E-07	3.57059	3.57E+00

Gene	p-Value	Fold Change (Male vs Female)	Ratio (Male vs Female)
IRF1	7.51E-09	2.37927	2.38E+00
CXCL14	1.95E-05	3.4451	3.45E+00
HLA-H	5.64E-05	2.33438	2.33438
CFB	1.26E-06	7.11924	7.12E+00
PSMB9	0.000199465	2.05177	2.05177
MYB	0.000101859	-2.25607	0.443249
UBD	0.000549427	4.18173	4.18E+00
TRIM31	0.00188772	2.71734	2.71734
TAP2	5.72E-10	3.06569	3.07E+00
PSMB8	7.57E-07	2.00503	2.01E+00
TAP1	3.68E-12	4.77759	4.78E+00
BAK1	6.43E-07	2.03942	2.04E+00
AKIRIN2	7.25E-05	2.23126	2.23126
VNN1	2.33E-06	2.07625	2.08E+00
C2	2.65E-05	3.02813	3.03E+00
C2	2.52E-05	2.9825	2.98E+00
C2	4.64E-05	2.75428	2.75E+00
C2	2.52E-05	2.9825	2.98E+00
HLA-A	4.98E-05	2.0267	2.0267
C2	2.60E-05	2.96166	2.96E+00
C2	2.58E-05	3.09803	3.10E+00
CD36	3.14E-07	-3.73752	2.68E-01
SLC7A2	5.30E-05	-3.38513	0.29541
CLU	8.42E-05	-2.243	4.46E-01
ENPP2	3.21E-05	-2.33398	0.428452
LY6D	1.01E-05	3.2538	3.25E+00
CD274	1.08E-09	5.85351	5.85E+00
PDCD1LG2	4.36E-06	2.81719	2.82E+00
LCN2	3.50E-07	6.69306	6.69E+00
IFNB1	1.22E-11	3.87632	3.88E+00
DDX58	9.96E-12	5.48656	5.49E+00
TRIM14	2.42E-06	2.19151	2.19E+00
TNFSF15	1.50E-05	2.01659	2.02E+00

Figure 6

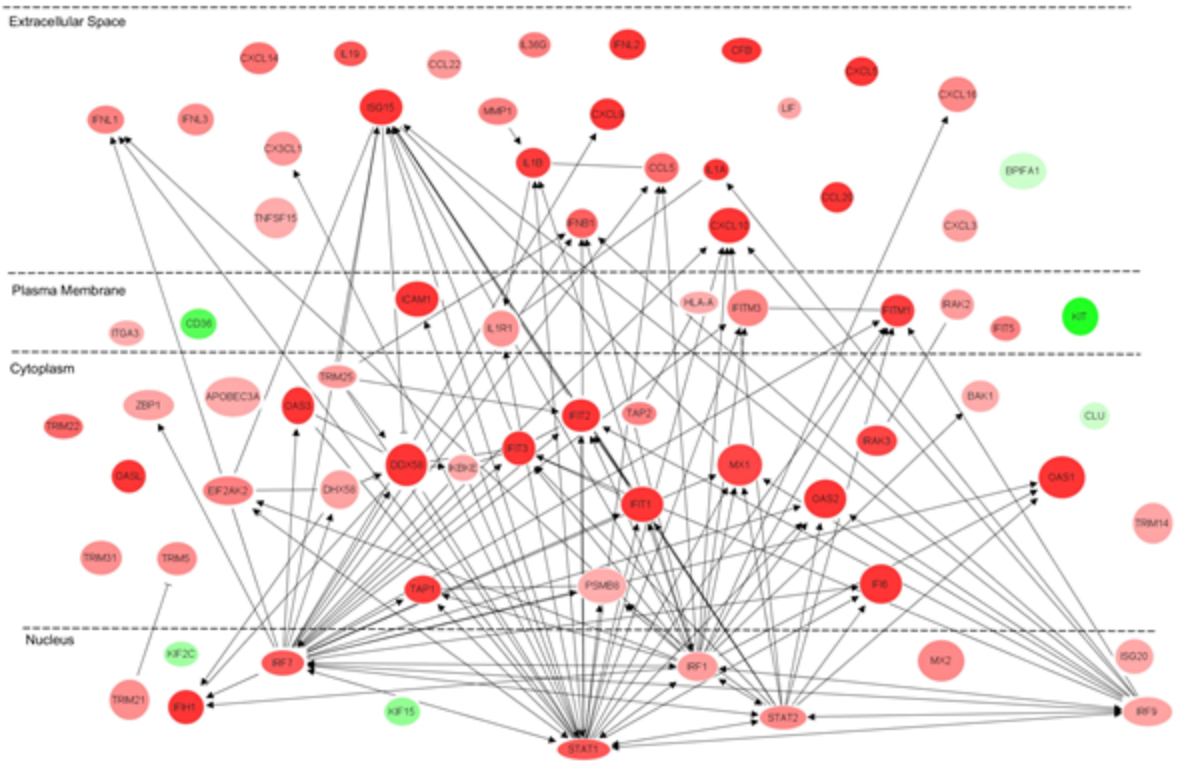


Figure 6: Genes along pattern recognition receptor and antiviral pathways were differentially expressed in hNECs from male versus female donors at 48 hours post rUdorn α2,3-infection. Using the list of 137 genes that were differentially expressed between the sexes 48hpi (Table 2), we imported the gene list into Ingenuity Pathway Analyses to identify pathways and the biological relationships between differentially expressed genes (B). Significant upregulation (127.75 to 2.00 fold change) in hNECs from males is illustrated in gradations of red and significant downregulation (-2.06 to -7.69 fold change) in males is indicated by gradations of green. Overall, hNECs from male

donors had a significantly greater number of upregulated genes than their female counterparts.

DISCUSSION

Conclusions

In this study, the donor sex of hNECs affected the host response to rUdorn α2,3-infection. While the donor sex of hNECs did not affect viral replication titers, it did influence the host cellular response to infection including production of IFN-λ and chemokines and the transcriptional activity of antiviral genes in hNECs. Overall, hNECs from males demonstrated higher antiviral activity after infection with rUdorn α2,3 IAV than their female counterparts.

There was no effect of donor sex on rUdorn α2,3 replication in hNECs, which is consistent with what has been reported in mouse studies of influenza A virus infection (Robinson et al., 2011). This result is counter to studies of other viruses such as HIV and Hepatitis B virus (HBV), which report differences in virus replication based on sex. Sex differences in HIV and HBV replication have been reported previously with Farzadegan and colleagues (1998) demonstrating that the HIV viral load in plasma is lower in female patients than males with the same CD4 T cell count. Despite differences in viral titers, the time to AIDS is the same for men and women after adjusting for CD4 T cell counts. Sex differences in plasma viral load and progression to AIDS is reported in other studies (Sterling et al., 2001), further confirming that HIV replicates differently in men and women resulting in differential progression of disease. Similar to HIV infection, men infected with HBV have greater viral titers in their sera than their female counterparts (Wang et al., 2009). Additionally, in studies of individuals with HBV, men and post-

menopausal women tend to progress through the stages of cirrhosis faster than infected women of reproductive age (Wang et al., 2009). In both HIV and HBV, males have greater viral titers in their sera than females yet females have the same or more rapid disease progression.

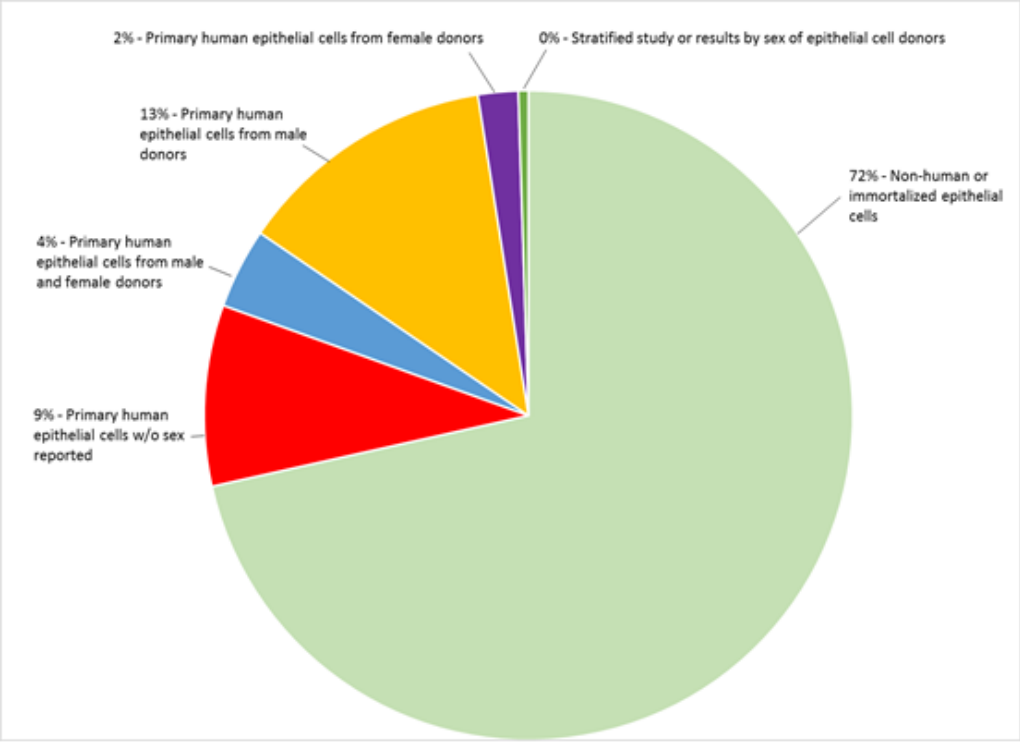
Sex differences in cellular responses to viruses have also been noted. For example, HIV replication is higher in peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (MDDCs) from male then female donors of reproductive age (25-45). Furthermore, HIV and HBV cause chronic infection (Farzadegan et al., 1998; Wang et al., 2009), whereas IAV causes an acute infection (WHO, 2015), meaning the kinetics of virus replication are different. Whether sex differences are more apparent in response to chronic viral infection as opposed to acute viral infections should be considered. Additionally, where within the cell these viruses replicate could be another factor influencing sex-specific outcomes.

Sex differences in antiviral responses to virus have been shown previously. For example, Griesbeck and colleagues (2015) stimulated plasmacytoid dendritic cells (pDCs) from healthy male or female donors with an HIV TLR7/8 ligand to examine the percent of pDCs producing IFN-α . They found that a significantly higher percentage of IFN-α producing pDCs were present following HIV stimulation in females than males (Griesbeck et al., 2015). In disease models, progesterone has been shown to increase IFN-α secretion by pDCs in response to HIV-1 derived TLR7/8 ligand stimulation (Meier et al., 2009). Studies utilizing RNA isolated from T-cells or DCs show that the expression of genes such as MX1 and CCR5 involved in TLR recognition of HIV infection and IFN-α

pathway are higher in females than males, which is associated with higher levels of immune activation (Chang et al., 2013). Finally, studies of pDCs from human male and female donors have shown that basal levels of IRF5, a mediator of TLR7 signaling, is higher in females than males which correlated with the percentage of INF- α secreting pDCs (Griesbeck et al., 2015). In our study females produced higher levels of IFN-λ, which is the main interferon produced in epithelial cells. Higher production of IFN-α by immune cells and greater production of IFN-λ by hNECs occur in cells from female as compared with male donors. While females produced higher levels of IFN-λ, they had lower levels of transcriptional IFN-λ activity. This may be due to the immunosuppressive role of testosterone. Males may need to upregulate IFN-λ transcriptional activity to a greater extent than their female counterparts in order to overcome the immune suppression on IFN-λ from testosterone.

In 2014, the National Institutes of Health (NIH) announced policies in regards to sex as a variable in preclinical research (Clayton & Collins, 2014). Prior to this mandate, there had been numerous studies that demonstrated a sex difference in disease or treatment outcome both in cell culture (Mittelstrass et al., 2011; Penaloza et al., 2009) as well as animal models (Du et al., 2014) yet many researchers were not aware of the sex of their cells or were failing to report them (Pollitzer, 2013). Since October 2014, researchers applying for NIH funding must now address how they plan to include male and female cells and animals in their research protocols. Additional funding and oversight committees have also been introduced to help with this transition and to ensure the compliance of researchers to the new guidelines (Clayton & Collins, 2014).

For the purposes of my project, I was interested in examining the reporting of the sex of epithelial cells used in IAV research. I conducted a literature search using PubMed with the search input, “in vitro influenza infection epithelial cells.” Of the 395 results, I examined 120 articles that were all published between 2010 and 2016. Of the 120 articles, only 34 reported the use of human epithelial cells in their work as opposed to epithelial cells derived from either animals or immortalized cell lines. Furthermore, of those 34 articles, only 5 reported using cells from both male and female donors. The most important result of this literature review was the discovery that none of the articles that I reviewed considered sex as a variable when conducting their research or stratified their results by sex. This result underscores how little is currently known about how sex affects the



pathogenesis of IAV in primary cell cultures, specifically epithelial cells. It also demonstrates how important it is to conduct studies such as mine because there is not much known about how sex influences IAV infection in these primary cell culture systems.

Future Directions

The investigation of the mechanism accounting for the observed sex differences in immune factor production and transcriptional activity between hNECs from male and female donors would be the next logical experimental step. In order to do this, an A/Udorn/72 NS1 mutant could be used to help investigate if the observed sex differences are occurring because of something the virus is inhibiting versus how the cells are responding to infection. Viral NS1 has several mechanisms by which it interferes with the host response to IAV infection including preventing type I IFN transcription and downregulating host gene expression via a complex with CPSF30 (Wang et al., 2000; Randall & Goodbourn, 2008; Nemeroff et al., 1998). If we repeated the viral replication, chemokine/cytokine production, and microarray analysis following infection with the NS1 mutant virus and the sex difference was still present, this would indicate that there is a sex difference in the response to IAV infection not a sex difference in which the virus inhibits cellular mechanisms in the host cell. If the sex difference I observed in my studies disappears using the NS1 mutant, this would

indicate that the sex differences is due to the virus inhibiting something in hNECs derived from female donors leading to the sex difference I observed.

Another potential experiment would be to look at the immunosuppressive role of testosterone in IAV infection. Previous work in our lab has shown that testosterone suppresses the innate immune response to infection with IAV in male mice. My study demonstrated chemokine production and transcriptional activity was higher in hNECs from male donors. I would like to investigate if this is due to a lack of circulating endogenous male hormones within the hNEC culture system that; within the nose epithelial cells may need to upregulate their transcriptional activity in order to overcome the endogenous hormones. If I were to treat my cells with testosterone and then infect them with IAV and see a dampening of their innate immune response, this may explain why my hNECs from male donors seemingly have a greater transcriptional and proinflammatory chemokine response to infection in my current system.

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Alisa Bochnowski

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PROFILE

Master of Science candidate with training in infectious disease identification, pathogenesis, risk, and management. Public health researcher with a strong background in laboratory sciences, literature review and data abstraction, and quantitative risk assessment.

EDUCATION

Master of Science (ScM) May 2016 Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Thesis work: *The Effect of Donor Sex on Influenza A Infection in Primary Human Nasal Epithelial Cell Cultures*

Bachelor of Science in Chemistry December 2013 The George Washington University, Washington, DC

PUBLIC HEALTH TRAINING

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD Aug. 2014 - Present

Public Health Emergency Preparedness (Spring 2016)

- Identified and described the main public health activities in preparing for and responding to public health emergencies
- Identified and examined possible chemical, biological, radiological, nuclear, and explosive terrorist weapons
- Described the role of public health agencies in emergencies, and interactions with public safety and other agencies

Crisis and Response in Public Health Policy and Practice (Fall 2015)

- Analyzed key elements of effective and ineffective day-to-day responses to crises at the local, state, national, and global levels
- Examined how public health leaders can manage existing crises effectively to win significant, long-term policy advances
- Identified the pros and cons of the use of deliberate language in a public health crisis

PUBLIC HEALTH/RESEARCH EXPERIENCE

- Analyzed data using appropriate statistical software such as SigmaPlot and Systat, presented data to a meeting of peers, presented data at a departmental meeting with senior faculty and investigators
- Critically reviewed and analyzed published research, extracted chemical compound data, toxicity data, and other pertinent data from infectious disease journal articles for inclusion in the NIAID AIDS database
- Developed a strategic plan for an organization, including a situational assessment and competitive analysis, identifying strategic options, and assessing and making appropriate plans and implementing the decided upon strategies
- Described the chemical properties and biological processes which modulate the toxicokinetics of chemical agents of public health importance, determined the molecular, cellular, and pathophysiological responses resulting from exposure to chemical agents relevant to human health, used toxicology principles in conducting a risk assessment, identified the elements of a quantitative risk assessment by utilizing the framework developed by the National Research Council
- Developed a thesis project looking at sex differences in pathogenesis of Influenza A infection in human nasal epithelial cells, including looking at viral replication kinetics, cytokine and chemokine production, and gene-level responses to infection

WORK HISTORY

Intern, Gryphon Scientific, LLC Takoma Park MD (Jan. 2014- June 2014

Student Researcher, National Institute of Allergy and Infectious Disease, Bethesda, MD (May 2013-July 2013)

Research Assistant, Optimantra, Washington, DC (Dec. 2012-Sept 2013)

Student Researcher, National Aeronautics and Space Administration, Greenbelt, MD **(**June 2012-Aug. 2012)

Tutor, The George Washington Tutoring Initiative, Washington, DC **(**Sept.2012-May 2013)

PUBLIC HEALTH EXPERIENCE

Generation Tomorrow, Baltimore, MD Sept. 2014-May 2015

- Worked with the Baltimore City Health Department to educate the community about HIV and Hepatitis C as well as provide testing and counseling services
- Provided community outreach and support through auxiliary activities such as World AIDS day and Testing for Turkeys

PROFESSIONAL DEVELOPMENT AND TECHNICAL SKILLS

Technical proficiency in: Mass spectrometry, Flow cytometry, Western blotting, Cell culture, ELISA, MSD assay, microarray

Computer Skills: Proficient in Word, Excel, Powerpoint, Access, Prism (graphical software), EndNote, FloJo, Ingenuity, Sigmaplot, Systat, research engines including Pubmed

Training: Certified HIV and Hepatitis C counselor and tester